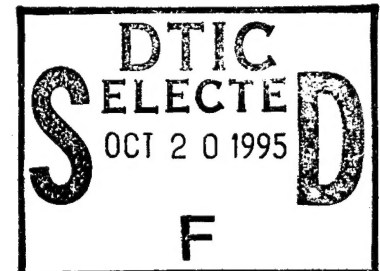


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BREAST DISEASE IN XENOGRAFT MODEL"

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P.V. Glatys Shikhan 8/29/95
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PROGRESS REPORT OF WORK ACCOMPLISHED IN YEAR ONE AND PLANS FOR YEAR TWO.

INTRODUCTION

Breast cancer is the most common malignancy among women in North America with the number of newly diagnosed cases increasing at a rate of about 1% every year. Although very little is known about the molecular events underlying the development of mammary tumors in humans, much evidence indicates that the presence of estrogen receptor (ER) in mammary carcinoma is generally a good prognostic marker because ER⁺ tumor cells are usually more differentiated and have lower metastatic potential. It is generally thought that hormones, including estradiol (E₂), act as tumor promoters by virtue of their effects on cell growth and differentiation, efforts of many investigators have focused on the mitogenic effects of E₂, or E₂ combined with progesterone. In rodent models, such studies have provided a great deal of information about the effects of hormonal status and pregnancy, or susceptibility of cells of the terminal duct lobules to carcinogens and tumor development, but there is some concern about the relevance of comparisons since cancers induced in rodents differ morphologically from human breast tumors (1). Only 6-10% of normal human breast epithelial cells are ER⁺, yet more than 60% of primary human breast tumors are ER⁺ and initially depend on E₂ for growth (2-4). This would suggest that: (1) ER⁺ cells are more likely to become transformed than ER⁻ cells and/or that the hormonal and cellular milieu of the breast is more conducive to growth of transformed ER⁺ cells or (2) if ER⁺ expression provides a growth advantage, the cells must undergo some additional change that allows them to avoid the growth inhibitory or differentiating effects of E₂. This would predict that in at least some cases, initially slow growing ER⁻ tumors could give rise to ER⁺ tumors with an increased potential for growth and progression (5).

In the human breast, a spectrum of microscopic changes has been termed proliferative breast disease (PBD). Although hyperplastic lesions are observed in human breast, their role in disease progression is not understood. The progression of histopathological features of PBD has been correlated with increased risk for the development of invasive carcinoma.

The aim of this study is to determine (a) the mechanism by which E₂ influences neoplastic progression of ER⁺ and ER⁻ human breast epithelial cells, and (b) whether stage-specific epigenetic and genetic changes occur during progression of precancerous human breast epithelial cells. We have utilized a xenograft model of early human breast cancer progression to address these questions. This model, a Ha-ras-transformed derivative of MCF10A (ER⁻, PR⁻) referred to as "MCF10AneoT", is a line of preneoplastic human breast epithelial cells which are able to grow in immunodeficient mice where they undergo a sequence of progressive histological changes culminating in cases of frank neoplasia in about 25% of the animals (6). Immortalized human breast epithelial MCF10A cells (7) are unable to survive long term in immune deficient nude/beige mice. Thus, MCF10AneoT and derivatives obtained from successive transplant generations (MCF10AneoT.TGn) provides a transplantable, xenograft model of human PBD with proven neoplastic potential. This model affords the opportunity to identify genetic and cellular changes that occur during early breast cancer development and to experimentally manipulate these changes to determine which alterations play a causal role in progression.

Alterations in relevant markers, viz., ER/PR expression, p53 expression/mutation, mdm-2 expression, DNA methylation status and genomic stability are being examined to determine if these correlate with progression. The effect of E₂ on neoplastic progression of human breast epithelial cells is being evaluated by comparing the effect of E₂ supplementation on progression of MCF10AneoT and MCF10AneoT derived cells in castrated male nude/beige mice.

Methods utilized include injection of cells into nude/beige mice, evaluation of lesions and cultured cells by histology and immunochemistry, in vitro culture of lesion-derived cells, analysis of p53 mutation by SSCP and sequencing. Genomic instability is being assessed by development of drug resistance; Southern blotting of appropriately restricted DNA to evaluate DNA methylation, and Northern blotting to evaluate gene expression.

BODY OF THE REPORT

Cell Culture:

MCF10A and MCF10A derivatives were maintained in phenol red-free DMEM/F-12 medium with 5% equine serum, 0.1 µg/ml cholera toxin, 10 µg/ml insulin, 0.5 µg/ml hydrocortisone, 0.02 µg/ml epidermal growth factor, 100 i.u./ml penicillin, 100 µg/ml streptomycin, and 5% horse serum. Charcoal-stripped serum was not used since it affects the proliferative ability and/or viability of MCF10A cells possibly due to removal of essential growth factors. Only serum that was not able to support growth of the ER⁺ cell line, MCF-7, (an indication that it was free of significant amounts of E₂) was routinely used.

Analysis of activated c-Ha-ras mRNA expression in MCF10A-derived cells:

Method: Two µg of total RNA extracted from MCF10A-derived cells were reverse transcribed to synthesize single stranded cDNAs using oligo (dT)₁₅ primers. Oligonucleotide primer set flanking the c-Ha-ras sequence from base 17 to 556 of the human Ha-ras cDNA (corresponding to base 1662-3317 of the human c-Ha-ras gene) were used for amplification by the polymerase chain reaction (PCR). The primers were sense, 5'-CGATGACGGAATATAAGCTGGTGGTGGT-3', and antisense, 5'-GGGGCCACTCTCATCAGGAGGGTTCAGCTT-3'. PCR products were cleaved with NaeI. All single base ras-activating substitutions at codon 12 eliminate a NaeI site (GCC/GGC) and give a novel 179 bp fragment under these conditions. NaeI-restricted cDNAs were analyzed by electrophoresis through 2% agarose gels.

Results: The novel 179 bp fragment released from NaeI digestion was observed only in the DNAs isolated from MCF10AneoT and the transplant generations indicating the presence of mutant Ha-ras only in the T24 ras-transfected cells (Fig. 1).

Analysis of normal and activated Ha-ras protein expression:

Method: MCF10A and MCF10A derivatives were rinsed in phosphate buffered saline (PBS) and resuspended in lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100). Lysate volumes equivalent to 50 µg of total protein were incubated with Ha-ras monoclonal antibody (clone Y159, Oncogene Science) followed by the addition of Protein G Sepharose. Bound Ha-ras was extracted from the Sepharose by boiling in Laemmli's buffer and

separated on a 12.5% polyacrylamide gel. The separated proteins were electroblotted onto immobilon membrane, incubated with Ha-ras monoclonal antibody, washed 4 times and then incubated with goat anti-mouse alkaline phosphatase conjugated antibody. The membrane was washed 4 times and detection was accomplished using the enhanced chemiluminescence detection kit (Clontech Labs. Inc., Palo Alto, CA).

Results: A 5-7 fold increase in the expression of total c-Ha-ras (wild type and mutant) was detected in MCF10AneoT and the transplant generations, MCF10AneoT.TG1, MCF10AneoT.TG2b and MCF10AneoT.TG3b as compared to MCF10Aneo, MCF10AneoN and the parental MCF10A cells (Fig. 2). As the monoclonal antibody, clone Y159, does not distinguish wild type from mutant Ha-ras, it is not clear whether the increased levels of c-Ha-ras observed in MCF10AneoT and its transplant generations are a result of overexpression of mutant Ha-ras or a combination of wild type (endogenous) and mutant (transfected) Ha-ras. Our results suggest that the failure to produce persistent lesions by MCF10AneoN, MCF10Aneo and MCF10A cells may be the result of low levels of Ha-ras expression. This would mean that either (a) a certain threshold level of Ha-ras may be required to promote neoplastic progression or (b) that mutant Ha-ras plays a major role in promoting neoplastic progression. The former possibility appears to play a more important role as mutation of the Ha-ras gene has not been observed in human breast cancer, instead overexpression of normal Ha-ras protein is frequently observed.

Detection of ER mRNA expression:

Method: Two µg of total RNA extracted from MCF10A-derived cell lines were reverse transcribed as described previously. Oligonucleotide primer set flanking the human ER cDNA sequence from base 276 to 1241 of ER (as numbered by Green *et al*, ref.8) which corresponded to exon 1 through exon 6 of ER were used for amplification by PCR. The primers were sense 5'-GCCCCGCGCCACGGACCATGACCAT-3', and antisense, 5'-TGACCATCTGGTCGGCCGTC-3'. The identity of the 966 bp ER cDNA fragment amplified from MCF10A derivatives was confirmed by Southern blot hybridization analysis with a full-length human ER cDNA probe. RT-PCR products were gel purified and cloned into TA-cloning vector (Invitrogen), and the double stranded plasmid DNA containing the cDNA inserts was alkali denatured and both strands sequenced using ER specific primers. The sequences were compared to those reported in the Gene sequence Data Bank (EMBL/GenBank).

Results: Figure 3 shows the presence of the 966 bp ER fragment amplified by RT-PCR only in MCF10AneoT and the transplant generations. MCF10A parental cells do not express ER mRNA. The 966 bp RT-PCR products amplified from MCF10AneoT and MCF10AneoT.TG3c were cloned into TA-cloning vector and sequenced. Of the 4 clones derived from MCF10AneoT cells, only one clone exhibited an alteration at codon 286 (in the hinge region of the ER molecule) in which methionine is replaced by threonine (ATG⇒ACG, data not shown). All 4 clones derived from MCF10AneoT.TG3c and 3 clones from MCF10AneoT showed sequences that corresponded to sequence established for human wild type ER.

Immunodetection of ER:

Analysis of ER expression:

Method: MCF10Aneo, MCF10AneoT, MCF10AneoT.TG3c and MCF-7 cells were rinsed in phosphate buffered saline (PBS), harvested from semiconfluent flasks, pelleted and resuspended in a high salt buffer of 10 mM NaMoO₄, 10 mM Tris, 1 mM EDTA, 1 mM DTT and 0.6 M KCl, pH 7.5. Cytosol fractions recovered from the cell lysates were incubated with human ER monoclonal antibody, D547, followed by the addition of Protein A Sepharose. Protein equivalent to 100 µg (MCF-7) and 200 µg (MCF10AneoT, MCF10AneoT.TG3c, MCF10Aneo) were used for immunoprecipitation. Bound ER was extracted from the Sepharose resin by boiling in Laemmli's buffer and separated on a 7.5% SDS-polyacrylamide gel. Proteins were electroblotted onto immobilon membranes, incubated with anti-ER mAb D547 and tagged with an ¹²⁵I-labeled anti-rat IgG. Following washes, the membrane strips were exposed to Kodak XAR film at -70°C for 1-3 days. The relative amounts of ER expressed were quantified by densitometry.

Results: MCF10AneoT and MCF10AneoT.TG3c expressed ER protein with molecular weight similar to that detected in MCF-7 cells. Only normal ER (Mr ~67 KDa) was detected and the levels were 4 and 6-fold lower in MCF10AneoT.TG3c (Fig. 4, lane 2) and MCF10AneoT (Fig. 4, lane 3), respectively, as compared to expression levels in MCF-7 cells (Fig. 4, lane 1). Please note that the amounts of protein from MCF10A derivatives loaded on the gel were twice the amount loaded from MCF-7 cells. Note the absence of immunoreactivity with ER mAb in MCF10Aneo cells (Fig. 4, lane 4).

Immunocytochemical staining of ER:

Method: MCF10AneoT.TG3c and MCF-7 cells were fixed in buffered formalin, reacted with Abbott anti-ER mAb H222 or with normal rat IgG.

Results: Nuclear staining for ER was observed in MCF-7 cells and MCF10AneoT.TG3c cells. Controls, either without mAb H222 or with nonimmune rat IgG lacked any nuclear fluorescence (data not shown).

Flow cytometry:

Method: MCF10AneoN, MCF10AneoT, MCF10AneoT-TG3c and MCF-7 cells were harvested and washed with PBS. The cells were fixed with methanol/acetone (1:1) for 30 min and then washed with PBS twice. The fixed cells were incubated for 60 min on ice with D547 ER monoclonal antibody or nonimmune rat IgG (isotype control) as a negative control. The cells were washed twice with PBS and then incubated with a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-rat IgG for 45 min. The stained cells were analyzed using a FACScan flow cytometer (Becton-Dickinson).

Results: Data from flow cytometry indicate approximately 2-fold higher levels of ER in MCF-7 cells as compared to MCF10AneoT and MCF10AneoT.TG3c cells, whereas no immunoreactivity was detected in MCF10AneoN cells (Fig. 5).

Although ER expression was detected in MCF10AneoT cells by Western blotting and flow cytometry, we have not been able to localize it reproducibly by immunocytochemical

staining. Similarly, immunocytochemical staining of ER in MCF10AneoT.TG3c cells has not been reproducible. ER positive TG3c cells (40%) were detected in two separate experiments, but were absent in three others. One of the reasons for this discrepancy could be the monoclonal antibody used for immunodetection. The monoclonal antibody, D547, was used for Western blotting and flow analysis, whereas monoclonal antibody, H222, was used for immunocytochemical detection. We will repeat immunocytochemical staining with D547 ER antibody as soon as it is available.

Determination of functionality of activated endogenous ER:

Method: Cell culture and transfection: MCF10AneoT cells (500,000 per 60 mm dish) were plated 18 h prior to transfection in phenol red-free media. The cells were transfected using the calcium phosphate procedure. Cells were incubated with 10 µg of plasmid DNA, JA12 (containing 2 copies of ERE inserted into p-(37)TK-CAT, ref.9) per dish for 6 h, followed by a 3 min incubation with 15% glycerol. The cells were treated with 17 β-estradiol at various concentrations for 36-42 h.

CAT assay: Transfected cells were lysed with four freezing and thawing cycles in 0.25 M HCl, pH 7.5. CAT assays were carried out as described by Gorman et al (10) with reaction mixtures containing 100 µg of supernatant protein and 0.1 µCi of ¹⁴C-chloramphenicol (40-60 mCi/mmol, ICN Radiochemicals, CA). The assay tubes were incubated for 4 h at 37°C. Acetylated chloramphenicol was separated by thin layer chromatography and quantitated by liquid scintillation counting. Protein concentrations were determined by the Bradford method. The pCH110 plasmid containing a functional lacZ gene (Pharmacia, New Jersey) was cotransfected in some experiments to monitor transfection efficiency and assayed according to the manufacturer's protocol.

Results: Figure 6 shows that ER protein expressed in MCF10AneoT cells is functionally active and is responsive to 17 β-estradiol. Co-transfection with pCH110 which expresses β-galactosidase activity at a constant level regardless of estradiol concentration, confirmed that the results were not due to differences in transfection efficiency or cell viability (data not shown).

Determination of methylation status of ER:

Method: Genomic DNAs isolated from MCF10Aneo, MCF10AneoT, MCF10AneoT.TG1, MCF10AneoT.TG2b, MCF10AneoT.TG3c and MCF-7 were digested with EcoRI and NotI. Cleaved DNAs were electrophoresed on 1% agarose gel, blotted and probed with a 0.3 kb EcoRI/PvuII fragment of plasmid POR3 radiolabeled with ³²P by random priming (11).

Results: Our results indicate that the NotI site in exon 1 of the ER gene is methylated in most cells in cultures of all MCF10A derivatives, whether they are ER⁻ or ER⁺, unlike in MCF-7 cells where it is hypomethylated (data not shown). Thus, loss of methylation at the NotI site is not necessary for ER gene activation. We have concentrated on examination of methylation of the ER promoter, since the question of regulation of expression of endogenous ER in these lines is of primary importance. We have switched to the technique of genomic sequencing at the CG rich regions in the promoter. We are completing the analysis of one particular CG rich region. So far our results indicate no methylation of CG sites in this region in DNA from MCF10A and MCF10AneoT cells but the pattern is quite different.

Evaluation of genomic instability:

Method: In order to determine to what extent introduction of T24 Ha-ras increases the genomic instability of parental MCF10A cells, and whether further increases in genomic instability occur in MCF10AneoT cells during passage through successive tumor generations (TG1, TG2, TG3, TGn), an indirect measure of genomic instability is being used. The capacity for development of resistance to N-(phosphonoacetyl)-L-aspartate (PALA) depends on the ability of a cell to amplify the gene coding for the multifunctional protein containing the enzyme activities, carbamoyl synthase, aspartate transcarbamylase and dihydroorotase (CAD). The method developed by Tlsty (12) has been successfully used to determine differences in genomic stability of cells from a Syrian hamster model of neoplastic progression (13). Plating efficiency and cell cycle times and LD50 for all cells is being determined in phenol red-free growth medium. Cells are seeded in appropriate numbers to give 2×10^5 to 2×10^6 cells/150 mm culture dish and selected for ability to form colonies at a stringency of 9 x the LD50 of PALA. A minimum of 2×10^8 viable cells are being examined for each population. A representative selection of at least 6 colonies are subcloned and reexposed to 9 x LD50 to assure true drug resistance.

Results: We have determined with a high degree of certainty that the amplification rate of the CAD gene in MCF10A cells is 10^{-4} . In MCF10AneoT cells this is increased 3 fold to 3×10^{-3} . Although statistically significant, it is probably not meaningful in terms of "stability" since both lines have an amplification frequency in the range of other transformed cells. In contrast, SCM10 cells, an early passage of MCF10A, have not given rise to any PALA resistant colonies from 10^7 cells examined. Interestingly, the same is true for MCF10AneoT.TG4J, derived from a TG3c lesion, which supports our colleague, Dr. Fred Miller's, observation that a stable clone with low frequency of progression has been selected by in vivo passage (personal communication). Work is in progress on the other clones and one experiment has been completed with MCF10AneoT cells grown in the presence of phenol red. The frequency of amplification was 10^{-4} , lower than cells grown in the phenol red-free medium used in all other experiments. This needs to be repeated but the data suggest that even low levels of estrogenic compounds may select for a more normal phenotype.

Analysis of p53 protein expression:

The importance of mutations in the coding regions of the p53 gene to development of breast cancer is underscored by the identification of such mutations in 60-70% of human breast carcinomas tested. A number of human breast cancer cell lines have been shown to express only the mutated form of p53. Deletions of 17p (which may include the *TP53* locus) with loss of heterozygosity have been noted in ~60% of breast tumors. Monoclonal antibodies prepared against several regions of the p53 protein have been used to identify overexpression and nuclear localization of the protein in both in situ and invasive carcinoma of the breast. These studies suggest that p53 mutations may be an early and consistent event in breast carcinoma induction.

Previous work from our laboratory has shown that MCF10A cells express a conformationally altered P53. However, we do not know whether a mutation has occurred that affects function. We have carried out the initial characterization of p53 mRNA and protein expressed by the MCF10AneoT system to determine whether alteration in p53 function could

have contributed to evolution of the morphologically normal human breast epithelial cells of the MCF10AneoT model to atypical and malignant forms.

In order to analyze P53 protein expression, we used monoclonal antibodies pAb421 (detects both wild type and mutant forms) and pAb240 (exclusively recognizes conformationally altered P53) (14).

Method: Exponentially growing MCF10A and MCF10AneoT-derived cells were incubated in methionine-free DMEM with 2% dialyzed fetal bovine serum supplemented with 100 μ Ci of 35 S-methionine (specific activity 1083 Ci/mmol, New England Nuclear). Monolayers were gently washed twice with PBS and lysed with 400 μ l of lysis buffer (150 mM NaCl, 10mM Tris-HCl, pH 7.5, 1% Triton X-100, 1 mM PMSF) at 4°C. Cell lysates were cleared by centrifugation for 15 min at 10,000 g and used immediately for immunoprecipitation. Lysate volumes containing equivalent amounts of radiolabel incorporated into TCA insoluble material (10^7) cpm were immunoprecipitated by incubating overnight at 4°C with 1 μ g of monoclonal anti-P53 antibody (PAb421 or PAb240, Oncogene Science) or normal mouse IgG. Protein G Sepharose beads were pelleted by centrifugation and washed four times in lysis buffer. Bound proteins were solubilized in SDS buffer and loaded on 8.5% polyacrylamide gels. After electrophoretic separation, gels were processed for fluorography, and subjected to autoradiography.

Results: Immunoprecipitation of metabolically labeled P53 with pAb421 showed the presence of similar amounts of wild type P53 in all MCF10A and MCF10AneoT derivatives. However, an increase in the proportion of the conformationally altered form (rapidly migrating form) of P53 was observed with each transplant generation (Fig. 7). This result was corroborated by immunoprecipitation experiments using the P53 antibody, PAb240 (data not shown). Recent studies by Zhu *et al* have demonstrated that wild type P53 can assume two different interchangeable conformations: the altered form is exclusively recognized by PAb240 but not by PAb1620 (15). The latter antibody recognizes only the wild-type form and not the mutant form of P53. Conformationally altered forms of P53 may occur either as a result of mutation or denaturation of wild-type P53 (probably due to the action of autocrine growth factors). Accumulation of PAb240-reactive P53 (denatured wild type P53 or mutant P53) has been shown to promote growth whereas the PAb1620-reactive wild type P53 suppresses cell proliferation (15).

PCR-SSCP (Single strand conformation polymorphism analysis):

Method: Total cellular RNA (2 μ g/15 μ l) from MCF10A, and MCF10A-derived cells were reverse transcribed using oligo(dT)15 primer. An aliquot of the reaction mixture was subjected directly to PCR using primer a⁺, 5'-GTCAGTCCATGGAGGAGCCG-3', and primer t, 5'-TTATGGCGGGAGGTAGACTG-3', to amplify p53 cDNA. Wild type p53 cDNA from the plasmid, pC53-SN3, was also subjected to PCR with primers a⁺ and t-. PCR was performed in a 50 μ l reaction mixture containing 1 μ l of RT-PCR template, 40 ng of each primer, 10 nM of each dNTP, 5 μ Ci of α - 32 P-dCTP and PCR buffer. PCR was carried out for 35 cycles of 2 min at 95°C, 2 min at 59°C, and 3 min at 68°C. A 5 μ l sample of each reaction was subjected to electrophoresis on 1% agarose gel and radiolabeled p53 fragments visualized by autoradiography. A portion of the 32 P-labeled PCR products was digested with restriction

enzymes, *Ava*II, *Bsm*AI, *Bsm*I or *Mae*III and subjected to agarose electrophoresis on 2.5% gels.

For SSCP analysis, the restricted PCR products were diluted 9-fold in denaturation solution (95% HCONH₂, 20mM EDTA, pH 8) and heated at 95°C for 5 min. The denatured samples were electrophoresed through 6% polyacrylamide gels in 0.5XTBE at 4°C and 25 W constant power (16). Following electrophoresis, the gels were dried and subjected to autoradiography.

Genomic DNA isolated from MCF10A-derived cells and normal human placenta were subjected to hot start PCR using primers e⁺ (5'-GATGCTGTCCCCGGACGATATT-3') and f (5'-TTGGCTGTCCCAGAATGCAAGAA-3'). Similarly, RT products derived from MCF10A-derived cells were also subjected to PCR using primers e⁺ and f. These radiolabeled PCR products were then subjected to SSCP analysis as described earlier.

Results: Results from SSCP analysis indicate the absence of sequence alterations in p53 transcripts expressed in MCF10AneoT and transplant generations.

Our data indicate that the increase in accumulation of the altered form of P53 observed in the MCF10AneoT xenograft model is not a result of genetic alteration.

Expres-sion of mdm-2:

Mdm-2, a cellular oncogene product, has been shown to bind p53 and eliminate its ability to function as a transcription factor (17). Amplification of the mdm-2 gene has been detected in a wide variety of carcinomas and soft tissue sarcomas (18). Overexpression of mdm-2 will inactivate the wild type p53 transcription factor activity, possibly allowing for the selection of mutant p53. We have examined mdm-2 protein expression to see if the increase in expression of conformationally altered p53 protein correlates with overexpression of mdm-2 protein.

Method: Cell lysates from MCF10A and its derivatives were prepared as described before and 40 µg of total protein from each line was electrophoresed on 7.5% SDS-polyacrylamide gels. The separated proteins were subjected to Western blot analysis as described before. Mdm-2 antibody (Ab-1, clone IF2, Oncogene Science) was used and detection was accomplished by using the enhanced chemiluminescence detection kit as described before.

Results: Overexpression of mdm-2 protein (2-fold) was detected in the MCF10AneoT and transplant generations as compared to MCF10AneoN, MCF10Aneo and parental MCF10A cells (Fig. 8).

CONCLUSIONS

Using a xenograft model for progression of human proliferative breast disease, we show that expression of the endogenous ER occurs only in MCF10A cells that are transfected with T24 Ha-ras. We have demonstrated ER expression by several procedures outlined in the 'Body' and have established functionality of the activated endogenous ER by transient CAT assays. The role of ER in gene activity and on differential stimulation of specific genes is currently being investigated. The presence of a functionally active ER makes this xenograft model very useful to

study the role of estrogen in neoplastic progression of human breast epithelial cells.

The role of the T24 Ha-ras gene in development of neoplasia in the MCF10AneoT system is not clear. Since Ha-ras mutations are rare in human breast cancer, it is possible that T24 Ha-ras mimics the effect of some more common, as yet unidentified, genetic defect. However, since normal Ha-ras overexpression is a property of both benign proliferative and malignant human breast tissues, it is possible that low level expression of mutated Ha-ras and high level of normal Ha-ras play the same role in maintaining growth potential of human breast epithelial cells in vivo. Our data seem to indicate that a certain threshold level of Ha-ras may be required for promoting neoplastic progression, and that MCF10AneoN cells fail to produce persistent lesions probably because the level of Ha-ras expression in these cells is very low. In order to achieve high levels of Ha-ras expression, we will transfect MCF10A cells with a construct that has the human wild type Ha-ras gene placed under the control of an inducible promoter. The Ha-ras-transfected MCF10A cells will be injected into nude/beige mice and the growth and progression of lesions will be followed.

The mechanism by which T24 Ha-ras is associated with the activation of ER in a ER⁻ line is not clear. However, preliminary experiments indicate that estrogen may play a role in driving high risk, preneoplastic lesions to carcinoma. Although, the number of animals used for the study is few, this conclusion is based on the incidence and progression of hyperplastic lesions in nude/beige mice injected with MCF10AneoT and its derivatives. The majority of lesions produced in mice injected with MCF10AneoT cells are simple or complex, irrespective of whether the cells are injected into male or female mice. To date, lesions formed by MCF10AneoT.TG1 have only been found to form carcinomas in female mice. However, the ratio of normal/hyperplastic lesions is the same for males and females indicating a possible effect of estrogen on progression from hyperplasia to carcinoma in females. This is supported by data from MCF10AneoT.TG3c experiments where there was no obvious difference in the distribution of hyperplastic lesion morphology related to the hormonal status of the host. The TG3c experiments also indicate that ER expression is not always sufficient for progression from hyperplasia to carcinoma, since ER⁺ TG3c cells do not progress to carcinoma in either males or females. Taken together these two experiments suggest that the hormonal status of the host has no effect on the incidence of lesions that progress to hyperplasia, but that in some tumor derived clones, ER mediated processes can increase the rate of progression of hyperplastic lesions to carcinoma (data from Dr. Fred Miller; data not shown).

The data obtained so far also indicate that genetic alterations in p53 are not responsible for neoplastic progression of the MCF10AneoT xenograft model. Factors affecting p53 function such as alterations in the signalling pathway of genes such as mdm-2, waf-1/cip 1 or gadd 45 may play a role in progression of this xenograft model for human proliferative breast disease. In fact our data indicate that overexpression of mdm-2 observed in MCF10AneoT derivatives may play a role in enhancing levels of conformationally altered p53, which could be one of the contributing factors for lesion progression.

Goals for year 2:

Work in progress:

1. To determine if estradiol influences the rate of progression of MCF10AneoT and MCF10AneoT.TG1: MCF10AneoT cells were grown in estrogen free media, and 10^7 cells were injected into each dorsal flank of 80 castrated male nude/beige mice. Half of these mice received implants of 1.7 mg (6 week release) E_2 , and the other half received similar implants carrying the vehicle. Lesions will be removed at 4, 12, 24 and 48 weeks. Two lesions will be selected at random from each test group for recovery of cells. Serial sections from the lesions will be examined immunohistochemically for expression of ER, PgR, p53 and mdm-2. Cells from these lesions will be examined for genetic instability and/or loss of p53 function, c-myc, mdm-2, ER and PgR expression. If the results indicate presence of functional p53, we will examine waf-1 and p16, genes that function downstream of p53.

2. Complete examination of CAD gene amplification in MCF10A lines grown in presence and absence of E_2 .

3. Complete examination of the ER promoter region. Although there are at least 5 regions of interest, the high cost of these sequencing experiments will probably limit us to two additional regions that contain the most regulatory motifs. We will examine HpaII methylation in c-myc, PgR and β -globin.

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Figure 1: Detection of T24 c-Ha-ras mRNA expression.

Lane 1, MCF10A; lane 2, MCF10Aneo; lane 3, MCF10AneoN; lane 4, MCF10AneoT; lane 5, MCF10AneoT.TG1; lane 6, MCF10AneoT.TG2b; lane 7, Molecular size markers. Note the novel 179 bp bands in lanes 4-6.

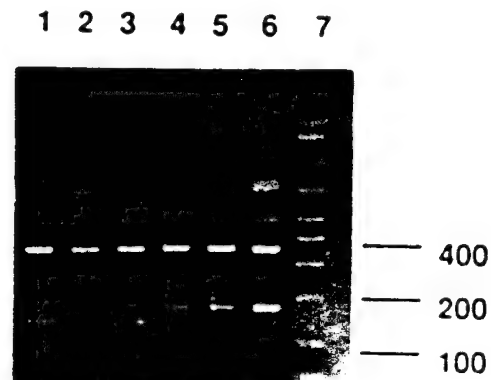


Figure 2: Analysis of normal and activated Ha-ras protein.

Lane 1, MCF10A; lane 2, MCF10Aneo; lane 3, MCF10AneoN; lane 4, MCF10AneoT; lane 5, MCF10AneoT.TG1; lane 6, MCF10AneoT.TG2b; lane 7, MCF10AneoT.TG3c.

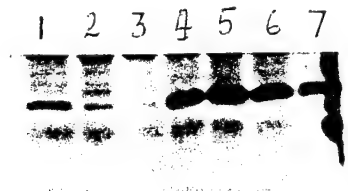


Figure 3: Detection of ER mRNA expression.

Lane 1, MCF10A; lane 2, MCF10Aneo; lane 3, MCF10AneoN; lane 4, MCF10AneoT; lane 5, MCF10AneoT.TG1; lane 6, MCF10AneoT.TG2b; lane 7, MCF10AneoT.TG3c; lane 8, ER cDNA amplified from a plasmid containing the full length human ER cDNA sequence. Note the presence of the 966 bp fragment amplified by RT-PCR only in MCF10AneoT and the transplant generations.

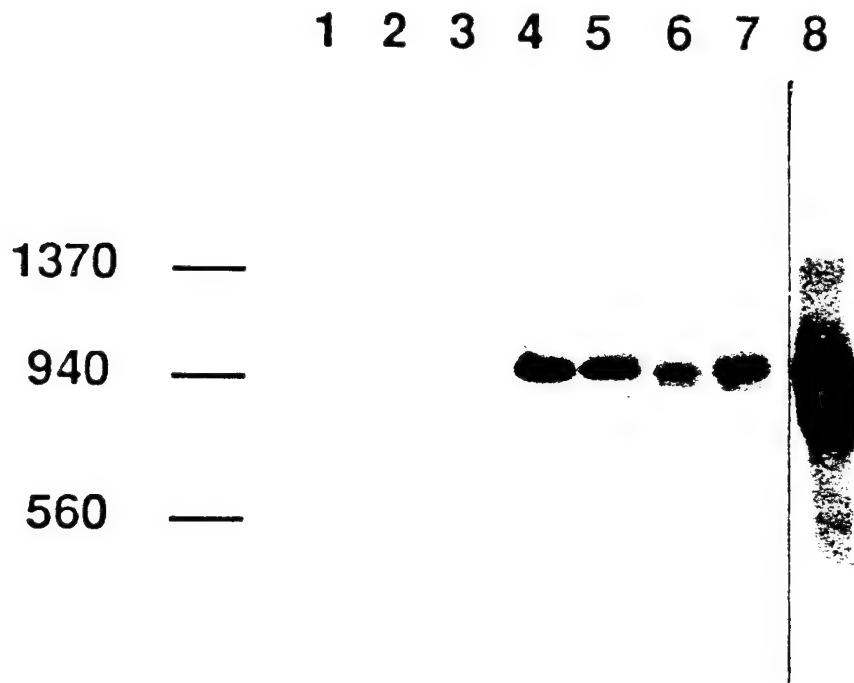


Figure 4: Analysis of ER protein in MCF10AneoT derivatives.

Lane 1, MCF-7; lane 2, MCF10AneoT.TG3c; lane 3, MCF10AneoT; lane 4, MCF10Aneo. Note the absence of immunoreactivity with ER monoclonal antibody in MCF10Aneo cells.

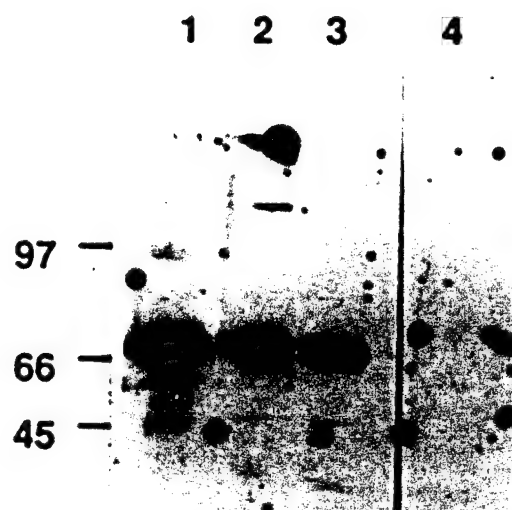


Figure 5: Analysis of ER expression by flow cytometry.

MCF10AneoN, MCF10AneoT, MCF10AneoT.TG3c and MCF-7 cells were reacted either with ER mAb, D547, or normal rat IgG, and stained with FITC-conjugated second antibody. The relative position of fluorescent labeled cells are indicated on a log scale. The mean peak values are also indicated.

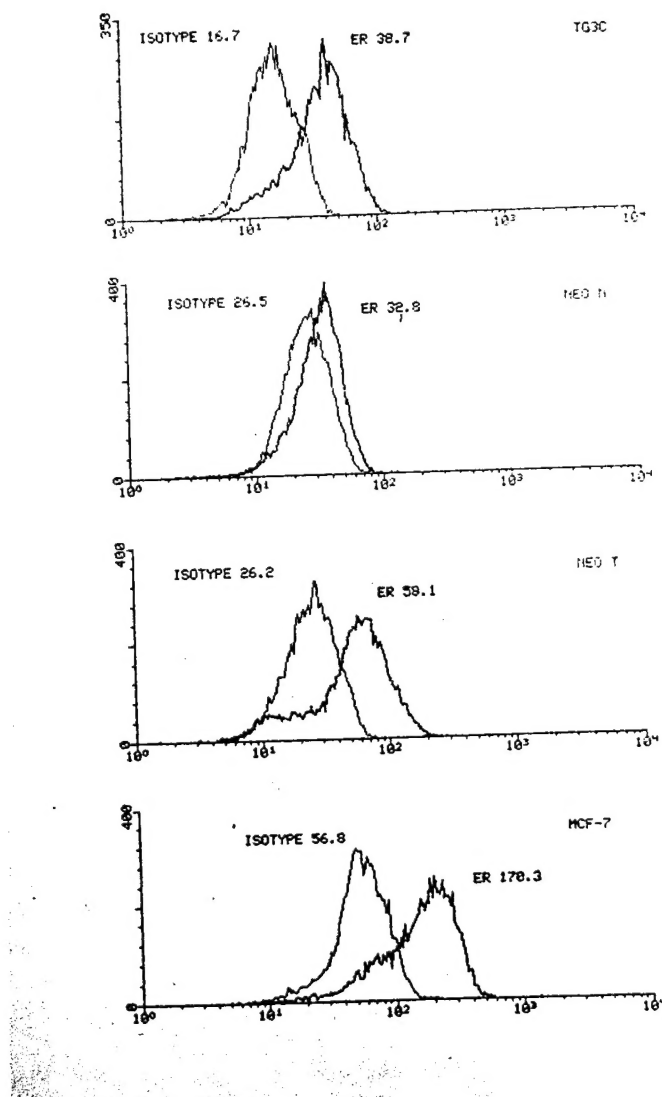


Figure 6: Induction of CAT expression by estrogen in MCF10AneoT cells. CAT activity induced in transfected MCF10AneoT cells in response to estradiol treatment is graphically represented. Co-transfection with pCH110 which expresses β -galactosidase activity at a constant level regardless of estradiol concentration, confirmed that the results were not due to differences in transfection efficiency or cell viability (data not shown).

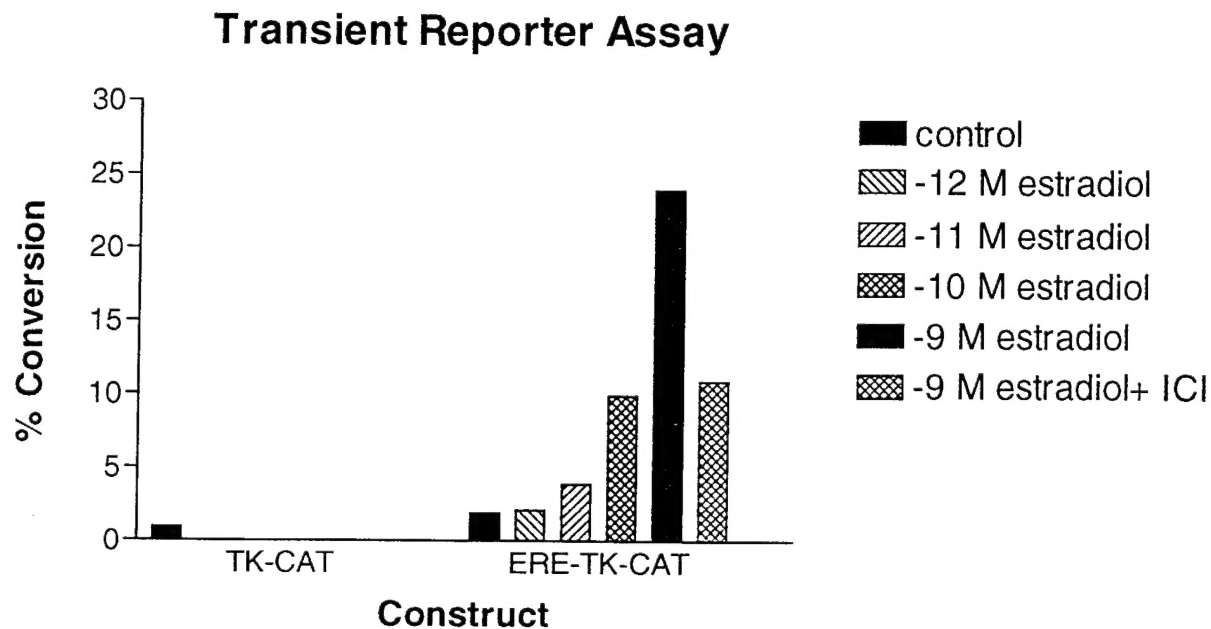


Figure 7: Analysis of p53 protein expression.

³⁵S-methionine labeled proteins were immunoprecipitated with monoclonal anti-p53 antibody, PAb421, and electrophoresed on 8.5% gels. Lane 1, labeled proteins of MCF10AneoT.TG2b immunoprecipitated with normal mouse IgG; lane 2, MCF10Aneo; lane 3, MCF10AneoN; lane 4, MCF10AneoT; lane 5, MCF10AneoT.TG1; lane 6, MCF10AneoT.TG2b; lane 7, MCF10AneoT.TG3c; lane 8, MCF10A.

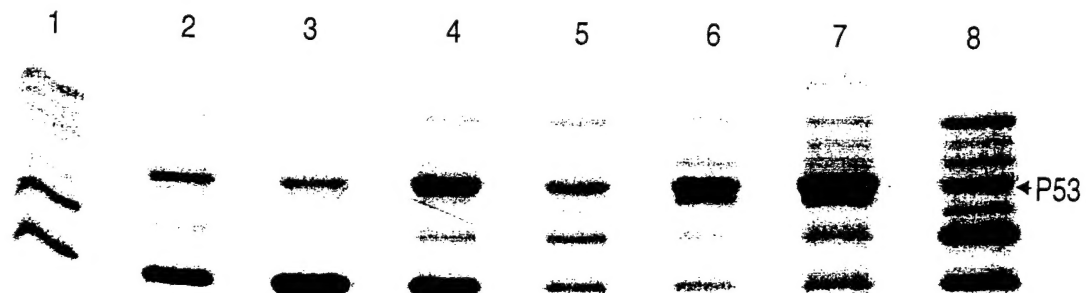


Figure 8: Western blot analysis of mdm-2 protein expression.

Lane 1, MCF10A; lane 2, MCF10Aneo; lane 3, MCF10AneoN; lane 4, MCF10AneoT; lane 5, MCF10AneoT.TG1; lane 6, MCF10AneoT.TG2b; lane 7, MCF10AneoT.TG3c.

